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Fate of antimicrobials and antimicrobial resistance genes in simulated swine manure storage

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HIGHLIGHTS

- Decay rates were determined for antimicrobials in anaerobic swine manure slurry.
- Decreases in *tet* and *erm* resistance genes were observed.
- Reductions in *tet* genes corresponded with reduced concentrations of chlortetracycline.
- Compounds in addition to parent antimicrobial may exert selective pressure for *erm* resistance.

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ABSTRACT

The behavior of three antibiotics (bacitracin, chlortetracycline, and tylosin) and two classes of antibiotic resistance genes (ARGs), *tet* and *erm*, were monitored in swine manure slurry under anaerobic conditions. First-order decay rates were determined for each antibiotic with half-lives ranging from 1 day (chlortetracycline) to 10 days (tylosin). ARGs were monitored in the swine manure slurry, and losses of approximately 1 to 3 orders of magnitude in relative abundance were observed during the 40 day storage period. First-order degradation profiles were observed for chlortetracycline and its corresponding resistance genes, *tet*(X) and *tet*(Q). Tylosin was degraded to approximately 10% of the starting concentration by day 40; however, the relative abundance of *erm*(B) remained at 50–60% of the initial relative abundance while the relative abundance of *erm*(F) decreased by 80–90%, consistent with tylosin. These results indicate that *tet* resistance genes respond primarily to chlortetracycline antimicrobials, and may be lost when the parent tetracycline compound is degraded. In contrast, *erm*(B) resistance gene may respond to a range of antimicrobials in animal manure, and may persist despite losses of tylosin.

1. Introduction

Antimicrobial resistance is among the world's most pressing public health concerns and the presence of both antimicrobial resistant bacteria and mobile antimicrobial resistance genes (ARGs) in the environment contribute to the evolution and spread of antibiotic resistance (Wellington et al., 2013). Wastes generated from animal production represent a major source of antimicrobials and ARGs to the environment (Pruden et al., 2013; Wang et al., 2012).

Antimicrobials are used in animal production at subtherapeutic levels for growth promotion and prophylaxis and at therapeutic levels for disease treatment. Antimicrobials added to animal feed are not completely absorbed during digestion, resulting in their presence in manure (Heuer et al., 2011). The presence and activity of antimicrobials in manure can select for antimicrobial resistant bacteria, even at low antimicrobial concentrations (Knapp et al., 2008) and antimicrobials, ARGs, and resistant bacteria can enter the environment through a variety of pathways including agricultural wastewater (Wantanabe et al., 2010; Zhang et al., 2013) or land applied animal manure (Zilles et al., 2005; Pei et al., 2006; Heuer et al., 2011; Joy et al., 2013).

Swine production in the United States was nearly 117 million heads in 2012 (USDA, 2013), and each animal can produce approximately 1500 kg of fresh manure by the time they reach market weight (Richert et al., 2005). Bacitracin A, chlortetracycline, and tylosin are antimicrobials commonly used in swine production (Cromwell, 2002; Jindal et al., 2006) and antimicrobial excretion rates of up to 90% in urine and 75% in feces have been reported (Halling-Sørensen et al., 2001). Swine produced at confined animal feeding operations (CAFOs) typically use one of three waste handling systems: flush systems, pit

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recharge, or deep pits (Sarmah et al., 2006). In deep pit systems, manure falls from a slatted floor into a pit below the animal housing facility and typically uses less water than either flush or pit recharge systems (Sarmah et al., 2006). Manure may be stored in these pits for up to a year. Deep pit systems are commonly used in colder climates such as the upper Midwest in the United States and manure accumulating in deep pits provides an environment for anaerobic microbial activities.

Relatively little information is available regarding the concurrent fate of antimicrobials and ARGs during anaerobic swine manure storage. Few studies have evaluated the fate of antimicrobials or ARGs in swine waste lagoons or storage pits. Evidence suggests that degradation of parent antimicrobials may occur during storage, but may not result in concurrent decreases in ARGs. Stone et al. (2009) found a 57% reduction in chlortetracycline concentrations and a 100% reduction in tylosin concentrations over 216 days in laboratory scale anaerobic batch experiments, where initial concentrations of chlortetracycline and tylosin were 28 and 1.1 mg/L, respectively. However, Chen and co-workers concluded that mesophilic anaerobic digestion and lagoon storage could not effectively reduce the absolute abundance of tetracycline and erythromycin resistance genes (Chen et al., 2010; Wang et al., 2012). Oxygen may also affect the fate of ARGs during swine manure storage. Diehl and LaPara (2010) tested the effects of oxygen and temperature on the degradation of ARGs in the biosolids of a wastewater treatment plant. They observed deceases in ARGs in anaerobic digesters under high temperature, while detecting no evident ARG decrease in aerobic digesters at the temperatures that were tested. Another study reported increases in ARGs during manure storage under aerobic conditions (Heuer et al., 2008). In that study, sulfonamide resistance genes *sul*(I) and *sul*(II) increased exponentially during the first 60 days of storage. Persistence of both antimicrobials and ARG in livestock manure determines subsequent entry into the environment through land application and the resulting potential for transport from agricultural watersheds.

The objectives of this study were to quantify the concentrations of three antimicrobials commonly used in swine production: bacitracin A, chlortetracycline, and tylosin, and their corresponding ARGs over time under simulated deep pit swine manure storage and to determine if the loss of the parent antimicrobial corresponds to decreased levels of antibiotic resistance genes in manure.

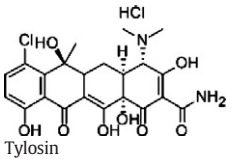
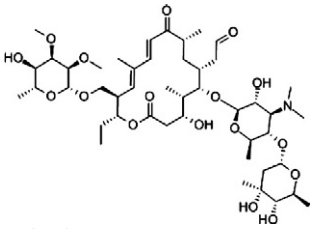
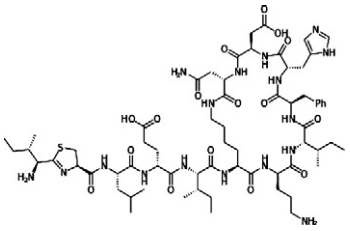
2. Methods and materials

2.1. Laboratory manure storage experiments

Swine manure was collected from the U.S. Meat Animal Research Center near Clay Center, Nebraska from separate barns where animals were administered bacitracin A, chlortetracycline, or tylosin. The chemical structure and physical–chemical properties of the antimicrobials can be found in Table 1. All animals were fed a corn and soybean-based diet with controlled dosages of antimicrobials and other supplements. Replacement gilts were fed 39.7 mg bacitracin A per kg feed, feeder pigs were fed 110.2 mg chlortetracycline per kg feed, and sows and gilts were fed 114.6 mg tylosin per kg feed. Fresh manure was collected directly from the floor in each animal housing unit utilizing one of the three target antimicrobials and transported to Lincoln, Nebraska where it was placed in experimental reactors. Additional properties of manure collected from the same facility are provided in Table 2.

100 mL glass amber wide mouth jars were used as sacrificial reactors. Manure and water were mixed in a 2:1 (w/w) ratio, and the homogenized mixture was allocated to each reactor for a total mass of 75 g (Masse et al., 2000). Reactors were sparged with nitrogen in an anaerobic chamber for approximately 5 min and incubated at 37 °C for up to 40 days. The reactor caps were briefly loosened every 1–2 days to prevent methane buildup within the reactors. Duplicate reactors were sacrificed at pre-determined periods. Samples were frozen at −20 °C until antimicrobial and ARG analyses were performed.

Table 1
Parent antimicrobial chemical structure and selected physical–chemical properties.[1]

Chlortetracycline 	Log K_{ow} = −0.62 K_d = 501–3715 L/kg (Teixidó et al., 2012) Solubility = 500 mg/L
Tylosin 	Log K_{ow} = 3.5 K_d = 1300 L/kg (Clay et al., 2005) Solubility = 6000 mg/L
Bacitracin A 	Estimated Log K_{ow} = −3.3 Estimated solubility = 1.5 μg/L

[1] Estimated properties are from US EPA EpiSuite Program v. 4.11.

2.2. Antimicrobial analysis

Antimicrobials were extracted from manure using solvent extraction followed by solid phase extraction (SPE) cleanup. Approximately 0.2 g of sample was mixed with 5 g of clean sand and spiked with 16 ng oleandomycin as a surrogate to monitor analyte recovery, followed by the addition of 14 mL of 5 mM ammonium citrate (buffered to pH 6 using ammonium hydroxide) and 6 mL methanol in 50-mL polypropylene centrifuge tubes. Mixtures were shaken briefly by hand and then on a Burrell wrist-action shaker for 30 min. Solids were extracted a second time with 4 mL of ammonium citrate and 16 mL methanol, and a third time using 20 mL acetone. All extracts were combined and fortified with internal standards (doxycycline and roxithromycin, 40 ng each) and then concentrated on a Labconco RapidVap N_2 sample concentrator (Labconco Corporation, Kansas City, MO) at 30 °C (90% rotation speed) until the volume was reduced by half. Purified reagent water was added to bring the extract volume to 100 mL and the resulting aqueous solutions were extracted using 200 mg Oasis HLB SPE cartridges. SPE cartridges were eluted into borosilicate test tubes using 3 mL of 130 mM ammonium citrate in methanol. The solvent was reduced in volume to approximately 200 μL under a stream of dry nitrogen, and transferred to an autosampler vial with silane-treated insert and then mixed with 200 μL reagent water. Roxithromycin was used as an internal standard for tylosin and bacitracin, while doxycycline was used for chlortetracycline. Recovery of bacitracin A, chlortetracycline, and tylosin, was determined from extraction and analysis of fortified reagent water during the elution stage. Fortified blanks and method blanks were analyzed at a frequency of 1 in 20 samples (5%). Method detection limits were determined by extraction and analysis of 8 replicates of reagent water samples fortified with antimicrobials at 0.005 μg/L. Method detection limits determine by extraction and analysis of 8 replicates of manure solids ranged from 0.5 ng/g for tylosin and chlorotetracycline, to 3 ng/g for bacitracin A and F. Recovery of target antibiotics as quantified by recovery of the surrogate were 95 ± 22% for chlortetracycline, 114 ± 29% for bacitracin, and 140 ± 68% for tylosin.

Table 2
Characteristics of swine manure.[1]

Manure animal treatment	NO_3–N (mg/kg)	NH_4–N (mg/kg)	NH_4–N:TN (Ratio)	Total N (mg/kg)	Total P (mg/kg)	N:P (Ratio)	Dry matter (%)	EC (dS m^{-1})	pH
Chlortetracycline	0.6 ± 0.4	788 ± 113	0.850 ± 0.08	940 ± 213	119 ± 66	9.34 ± 3.87	0.37 ± 0.13	7.82 ± 1.26	7.6 ± 0.3
Bacitracin A	0.9 ± 0.2	404 ± 93	0.555 ± 0.211	799 ± 252	219 ± 108	3.97 ± 0.90	0.84 ± 0.43	4.26 ± 0.70	6.8 ± 0.5
Tylosin	0.7 ± 0.3	441 ± 328	0.597 ± 0.057	770 ± 650	332 ± 263	2.37 ± 0.63	0.89 ± 0.74	4.36 ± 2.79	7.2 ± 0.3

[1] Reported as average ± standard deviation where n = 5. Values are reported in Gilley et al. (2013).

All sample extracts were analyzed on a Waters 2695 high pressure liquid chromatograph (HPLC) interfaced with a Waters Quattro Micro triple quadrupole mass spectrometer (Snow et al., 2003; Govaerts et al., 2003). Analytes were separated on a reverse phase (HyPurity C18, 250 mm × 2.1 mm, 5 μm particle size) column at 50 °C with a 50-μL injection volume. A gradient mobile phase (0.2 mL/min) was used consisting of A) 1 mM aqueous citric acid and methanol (97:3, v/v) and B) methanol and 1 mM aqueous citric acid (97:3, v/v). Initial gradient conditions (95% A) were held for 2 min, ramped to 5% A and held for 16 min., and then returned to 95% A for 5 min to equilibrate the column. Analytes were detected using Multiple Reaction Monitoring (MRM) mode with positive electrospray ionization (ESI). The most intense MRM transitions were determined by infusion and monitored for each analyte (Table 3) and linear calibration curves were generated for all analytes and surrogates with r^2 values of >0.995.

2.3. ARG analyses

DNA from storage samples was extracted using the MoBio Ultra-Clean Soil DNA Isolation Kit (Solana Beach, California) according to the manual except that a 40-second bead beating was used to lyse the cells. DNA extracts were quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE). Two *tet* genes were consistently detected in the chlortetracycline-manure from the same facility in this study and a previous study (Joy et al., 2013). qPCR conditions for *tet*(Q) and *tet*(X) were adopted from published studies (Ghosh et al., 2009; Koike et al., 2007). The preparation of qPCR standards for these two ARGs was reported in a previous study (Zhang et al., 2013).

Among the six *erm* (*e*rythromycin *r*ibosome *m*ethylase) resistance genes tested (i.e., *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(G) and *erm*(Q)) using PCR, *erm*(B) and *erm*(F) were detected in the tylosin-manure samples. The PCR products of *erm*(B) and *erm*(F) were purified using a QIAquick PCR Purification Kit, cloned and transformed using the TOPO® TA Cloning® Kit for Sequencing with One Shot® TOP10 (Invitrogen, Carlsbad, CA). Plasmids were extracted from the transformed *Escherichia coli* cells using Qiagen's Plasmid Midi Kit. The plasmid extracts containing target ARG amplicons were quantified using the NanoDrop spectrometer, calculated using a published equation (Li et al., 2012), and were diluted with sigma water to form a standard series. qPCR conditions for *erm*(B) and *erm*(F) were adopted from Koike et al. (2010).

Table 3
Molecular weight, retention times, and multiple reactor monitoring (MRM) transition of antimicrobials, internal standards (IS), and surrogate compounds (S).

Analyte	Molecular weight	Retention time (min)	MRM Transition (m/z)
Bacitracin A	1422.7	9.82	712.10 → 86.20
Bacitracin F	1419.64	10.05	710.19 → 281.26
Chlortetracycline	478.88	8.71	478.90 → 444.00
Tylosin	916.10	10.40	916.9 → 174.2
Doxycycline (IS)	444.4	8.63	445.05 → 428.05
Oleandomycin (S)	687.86	10.51	688.35 → 544.10
Roxythromycin (IS)	837.05	11.58	837.55 → 679.50

Regular PCR was also run on bacitracin-manure samples for bacitracin resistance genes *bceA* and *bceR* (Yoshida et al., 2011) as well as *bcrA*, *bcrB*, and *bcrC* (Murphy et al., 2008). All qPCR results on ARGs were normalized to the 16S rRNA gene, which was quantified using the qPCR protocol from Suzuki et al. (2000). All qPCR reaction were performed on a Mastercycler ep 147 realplex thermocycler (Eppendorf, Hamburg, Germany) using the RealMasterMix SYBR ROX qPCR kit (5 Prime, Gaithersburg, MD). Triplicate measurements were done on each DNA extract, and the averages of the triplicate measurements were used to calculate relative abundance values. The linear range, R^2, and amplification efficiency information for each qPCR protocol has been reported in one of our previous studies (Joy et al., 2013).

3. Results and discussion

3.1. Fate of antimicrobials during swine manure storage

The parent antimicrobials tylosin and chlortetracycline were detected in swine manure reactors at initial concentrations of 10 mg/kg (dry weight basis) and 300 mg/kg (dry weight basis), respectively (Fig. 1). Bacitracin A was not detected in the manure at any time, but bacitracin F, a metabolite of bacitracin A, was detected at an initial concentration of 50 mg/kg (dry weight basis) in the manure (Fig. 1). These reported concentrations are consistent with antibiotic concentrations measured in manure from the same facility in a previous study by the authors, where reported concentrations of chlortetracycline and tylosin in swine manure slurry were 404, and 32.5 mg/kg (dry weight basis), respectively (Joy et al., 2013). Other studies have reported chlortetracycline and bacitracin concentrations in swine manure as high as tens of mg/kg (dry weight basis) (Ji et al., 2012; Zhao et al., 2010; Martinez-Carballo et al., 2007) and 3.2–15 mg/kg (dry weight basis) (Zhou et al., 2013), respectively. Differences in reported antibiotic concentrations in manure may be due to the timing of manure collection, as antimicrobials can be sequestered (Wang et al., 2006) or degraded (Dolliver et al., 2008) during manure aging.

Observed antimicrobial concentrations were fit with a first-order decay equation to determine degradation rate constants and first-order half-lives (Table 4). A first-order expression fit the chlortetracycline and bacitracin F data well, but did not fit well for tylosin. The predicted degradation rate constant for tylosin was $-0.07\ day^{-1}$ ($R^2 = 0.34$), with a corresponding half-life of 9.7 days. The first-order degradation rate constant for chlortetracycline and bacitracin F were $-0.6\ day^{-1}$ ($R^2 = 0.79$) and $-0.36\ day^{-1}$ ($R^2 = 0.94$), respectively. Predicted first order half-lives were 1 day for chlortetracycline and 1.9 days for bacitracin F. The half-life for chlortetracycline measured in this study is shorter than that reported previously in studies of chlortetracycline degradation in swine manure or soil. Stone et al. (2009) did not report a first-order half-life for chlortetracycline for anaerobic swine manure slurry, however, based on the chlortetracycline concentrations reported in this study, a first-order half-life is estimated to be approximately 70 days. Chlortetracycline half-lives of 25–30 days (Li et al., 2010) and 20 days (Carlson and Mabury, 2006) have been reported for soil or compost under aerobic conditions. It is not clear why a faster chlortetracycline half-life was reported in this study when compared

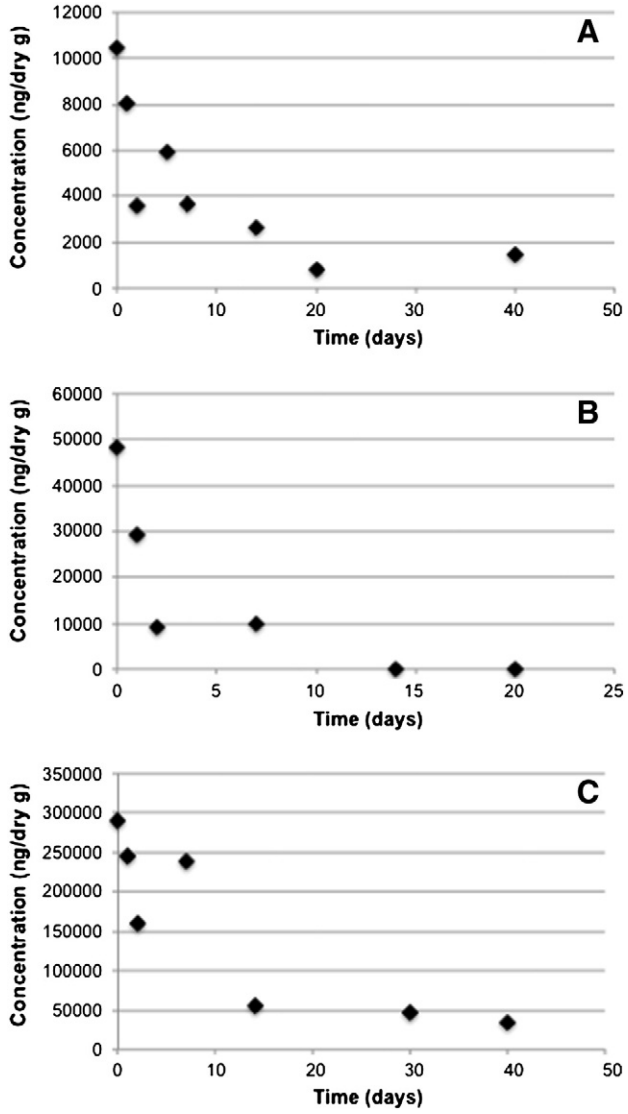


Fig. 1. Degradation of tylosin (Panel A), bacitracin F (Panel B), and chlortetracycline (Panel C). Concentrations expressed in units of ng compound per dry gram of manure.

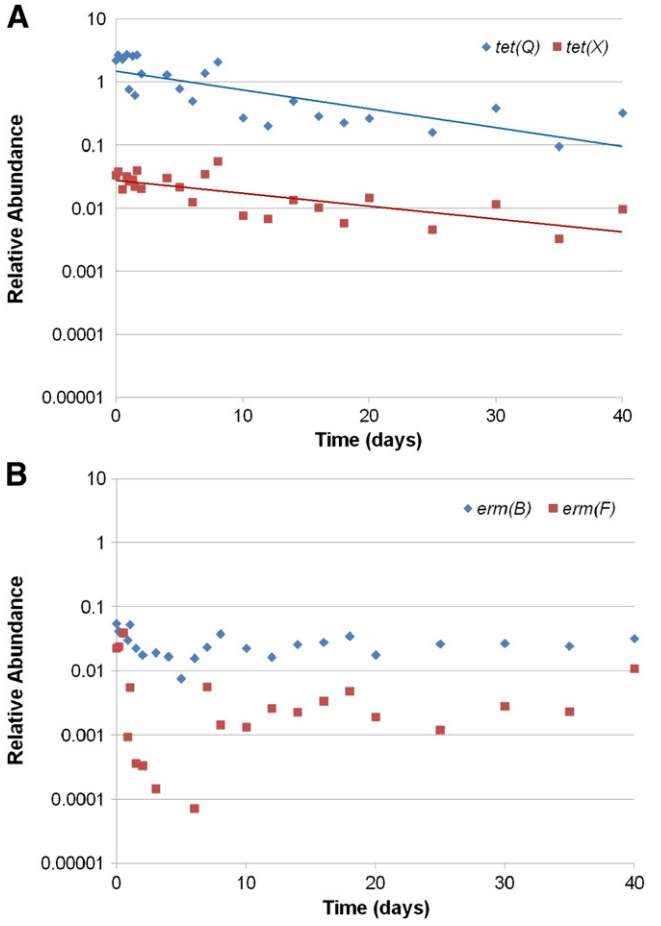


Fig. 2. Change in the relative abundance of antimicrobial resistance genes under simulated swine manure storage. Panel A shows results for *tet* genes while Panel B shows results for *erm* genes.

with previous studies, but could reflect differences in redox conditions or pH between the studies. Most previous studies that have reported chlortetracycline half-lives have investigated dissipation after spiking the compound into soil or manure (Li et al., 2010; Carlson and Mabury, 2006), in contrast to the present study where manure was collected from animals administered chlortetracycline. In contrast, the tylosin half-life reported in this study is consistent with previous studies that measured tylosin half-lives on the order of 4.4 days (Carlson and Mabury, 2006). Kolz et al. (2005) measured tylosin degradation in manure slurry under anaerobic conditions, and based on reported decay rates, tylosin half-lives in anaerobic manure ranged from approximately 0.5 to 2 h, shorter than the half-lives measured in the current study. We are not aware of previously published degradation rates for bacitracin F in swine manure.

3.2. Relative abundance of ARGs during storage

Tetracycline resistance genes *tet*(Q) and *tet*(X) and tylosin resistance genes *erm*(B) and *erm*(F) were detected in the chlortetracycline- and tylosin-manure reactors, while no bacitracin resistance genes were detected in the bacitracin-manure reactors. This is consistent with the results of a previous study performed by the authors (Joy et al., 2013). Over the 40-day degradation experiments, the relative abundance of ARGs followed a generally decreasing trend. The relative abundance of *tet*(Q) dropped by an order of magnitude over the course of the experiment (Fig. 2A). During the same period, the relative abundance of *tet*(X) decreased by less than one order of magnitude. The profiles of the two *tet* genes can be described using exponential decay trendlines (Fig. 2, $R^2 = 0.59$ for *tet*(Q) and $R^2 = 0.54$ for *tet*(X)). The relative

Table 4
Half-lives and degradation rates for antimicrobials measured in this study.

Antimicrobial	Measured degradation rate (day^{-1})	Measured half-life (day)	Reported half-life (day)
Chlortetracycline	−0.6	1	20–70 days (Carlson and Mabury, 2006; Stone et al., 2009)
Tylosin	−0.07	9.7	0.02–4.4 (Kolz et al., 2005; Carlson and Mabury, 2006)
Bacitracin F	−0.36	1.9	Not available

abundance of *tet*(Q) was consistently higher than that of *tet*(X) throughout the experiment (Fig. 2A). For tylosin resistance genes, the relative abundance of *erm*(B) dropped by less than one order of magnitude within the first 48 h of the experiment and then remained at the same level for the remainder of the 40-day experiment (Fig. 2B). The relative abundance of *erm*(F) dropped nearly three orders of magnitude in the first 48 h, but then increased about 1.5 orders of magnitude over the next 15 days before it leveled off (Fig. 2B). The relative abundance of *erm*(B) was consistently higher than that of *erm*(F) by one order of magnitude and the profiles of the two *erm* genes could not be satisfactorily described using simple trendlines.

Limited information is available to describe the effects of manure storage on the quantity of antibiotic resistant bacteria or ARGs in livestock manure (Chee-Sanford et al., 2009). The four ARGs detected in this study followed a general decreasing trend, although some variations were observed (Fig. 2). The phenomenon of different ARGs exhibiting different behaviors is not uncommon in manure or soil systems. Alexander and co-workers used qPCR to monitor the abundance of ARGs in cattle fecal deposits and their results showed that some ARGs (i.e., *tet*(B), *tet*(C), *sul*(I), *sul*(II), and *erm*(A)) first increased and then declined, while other ARGs (*tet*(M) and *tet*(W)) gradually decreased over 175 days (Alexander et al., 2011).

Fig. 3 shows the normalized change in antimicrobial concentration or ARG relative abundance as the ratio of the values at time t to the values at time 0 (C_t/C_o). Similar degradation profiles are seen for chlortetracycline and its corresponding resistance genes, *tet*(X) and *tet*(Q) (Fig. 3A). In contrast, while tylosin was degraded to approximately 10% of the starting concentration by day 40, the relative abundance of the *erm*(B) remained at 50–60% of the initial relative abundance while the relative abundance of *erm*(F) decreased by 80–90%, consistent with observed losses of tylosin (Fig. 3B).

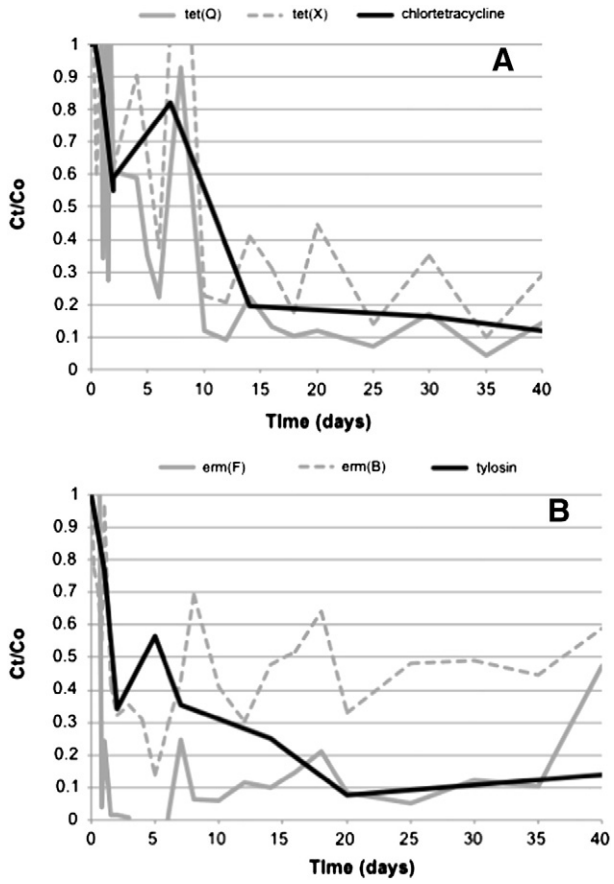


Fig. 3. Normalized changes in antibiotic concentration and ARG relative abundance in simulated swine manure storage. Panel A is chlortetracycline and *tet* resistance genes and Panel B is tylosin and *erm* resistance genes.

A variety of *erm* genes encode resistance phenotypes to macrolides, lincosamides, and streptogramins B (MLS_B), and *erm* proteins dimethylate a single adenine in the 23S rRNA, which is part of the large ribosomal subunit (Weisblum, 1995). As methylation occurs, binding of erythromycin, which is a macrolide, to its target is impaired. Because the binding sites of the MLS_B antimicrobials overlap, cross resistance caused by methylation occur to these three classes of antimicrobials (Leclercq, 2002). In this study, the concentration of tylosin, one macrolide antimicrobial, was quantified in manure samples. It is possible that other MSL_B antimicrobials also occurred in the samples and persisted through the experiment. This may explain why *erm*(B) did not decrease as much as tylosin (Fig. 3B). In contrast, the two *tet* genes tested in the study mostly respond to chlortetracycline. The *tet*(X) gene encodes an enzyme which modifies and inactivates the tetracycline molecule (Speer et al., 1991), while the *tet*(Q) gene encodes a cytoplasmic protein that protect the ribosomes from the action of tetracycline (Chopra and Roberts, 2001). The specificity of the *tet* genes might explain their overlapping trends with the chlortetracycline compound (Fig. 3). Further information regarding co-selection of individual ARGs by environmental conditions (e.g., presence of multiple antimicrobials) is needed to better understand how antimicrobial resistant bacteria that host these ARGs respond to environmental conditions (e.g. the presence of multiple antimicrobials).

4. Conclusions

In this study, the fate of antimicrobials and ARGs was monitored in anaerobic swine manure slurry over a 40-day period. All three antimicrobials monitored demonstrated losses over the 40-day period, with observed half-lives ranging from approximately 1 to 10 days. The half-life for chlortetracycline measured in this study was shorter than those reported in previous studies of anaerobic swine manure or aerobic soil and compost degradation. Results from this study also provide evidence that bacitracin A is rapidly degraded in the environment, and a half-life for one bacitracin A transformation product, bacitracin F, was determined to be approximately 2 days. This study represents one of the first investigations of bacitracin fate in the environment. The fate of the corresponding ARGs was also monitored. The degradation profile of *tet* genes was found to mirror that of chlortetracycline. The two *erm* genes exhibited distinctive degradation profiles: *erm*(F) relative abundance and tylosin concentration both dropped to approximately 10% of their initial values at the end of the 40 day period, while the relative abundance of *erm*(B) persisted at approximately 50% of the starting value over the study period. This indicates that tylosin degradation products or other MLS_B antimicrobials might exert selective pressure for certain *erm* genes. The mechanism responsible for this observed behavior is not currently understood. This study showed the importance of monitoring antimicrobials and ARGs over longer time periods to more fully evaluate their concurrent fate in manure storage systems.

Acknowledgments

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